Revisiting Cardiac Cellular Composition

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ABSTRACT

**Rationale:** Accurate knowledge of the cellular composition of the heart is essential to fully understand the changes that occur during pathogenesis and to devise strategies for tissue engineering and regeneration.

**Objective:** To examine the relative frequency of cardiac endothelial cells, hematopoietic-derived cells and fibroblasts in the mouse and human heart.

**Methods and Results:** Using a combination of genetic tools and cellular markers, we examined the occurrence of the most prominent cell types in the adult mouse heart. Immunohistochemistry revealed that endothelial cells constitute over 60%, hematopoietic-derived cells 5-10%, and fibroblasts under 20% of the non-myocytes in the heart. A refined cell isolation protocol and an improved flow cytometry approach provided an independent means of determining the relative abundance of non-myocytes. High dimensional analysis and unsupervised clustering of cell populations confirmed that endothelial cells are the most abundant cell population. Interestingly, fibroblast numbers are smaller than previously estimated, and two commonly assigned fibroblast markers, Sca-1 and CD90, underrepresent fibroblast numbers. We also describe an alternative fibroblast surface marker that more accurately identifies the resident cardiac fibroblast population.

**Conclusions:** This new perspective on the abundance of different cell types in the heart demonstrates that fibroblasts comprise a relatively minor population. By contrast, endothelial cells constitute the majority of non-cardiomyocytes and are likely to play a greater role in physiologic function and response to injury than previously appreciated.

**Keywords:** Endothelial cell, fibroblasts, leukocyte, flow cytometry, heart, SPADE.

**Nonstandard Abbreviations and Acronyms:**

<table>
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<th>Abbreviation</th>
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<tr>
<td>αSMA</td>
<td>α smooth muscle actin</td>
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<td>CM</td>
<td>cardiomyocyte</td>
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<td>EC</td>
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<td>EMP</td>
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<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>FSC-A</td>
<td>forward scatter-area</td>
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<td>GFP</td>
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<td>IB4:</td>
<td>isolectin B4</td>
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<td>LEC</td>
<td>lymphatic endothelial cell</td>
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<td>LV</td>
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<td>MEFs</td>
<td>mouse embryonic fibroblasts</td>
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<td>MHC class II</td>
<td>major histocompatibility complex class II</td>
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<td>RMC</td>
<td>resident mesenchymal cells</td>
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<td>SPADE</td>
<td>spanning-tree progression analysis of density-normalized events</td>
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<tr>
<td>VDO</td>
<td>Vybrant® DyeCycle™ Orange</td>
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<td>VEC</td>
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INTRODUCTION

While cardiomyocytes account for approximately 25-35% of all cells in the heart\(^1,2\), there is a lack of consensus regarding the composition of the cardiac non-myocyte cell population. In mammals, cardiomyocytes reside in close proximity to capillaries, and the calculated ratio of endothelial cells to cardiomyocytes is 3:1\(^3,4\). This estimated number of endothelial cells contradicts studies that have previously characterized heart cellular composition, where findings suggested that fibroblasts constitute the principal non-myocyte cell type\(^1,2,5,6\). Often these analyses relied on the expression of singular proteins for identifying specific cell types; this approach may have resulted in exclusion of or loss of distinct cell populations. Because of the incongruence between these data sets and recent advances in our knowledge of heart resident immune cells\(^7,8\), we revisited the issue of cardiac cellular composition. Using newly available genetic tracers and enhanced flow cytometry techniques to analyze the relative abundance of different cell types in the human and mouse heart, we document that fibroblasts comprise a relatively minor cell population and that endothelial cells are the most abundant cell type.

METHODS

**Cardiac single cell preparation.**

Mouse hearts were isolated for single cell preparation as previously described\(^9\) with atria and valves removed. Isolated mouse hearts were digested using one of three protocols (designated Protocol 1, 2 and 3). For Protocol 1, each mouse heart was divided in approximately 20 pieces, placed in 10 ml of Protocol 1 digestion buffer [2 mg/ml collagenase type II in 1X HBSS (Worthington Biochemical Corporation)] in gentleMacs C-tubes (Miltenyi Biotec), dissociated using *Heart 1* program of a gentleMacs Dissociator, and incubated at 37°C for 40 min with gentle agitation. Following incubation, tissue was further dissociated using *Heart 2* program before placing on ice. For Protocol 2, isolated hearts were finely minced using forceps to ~2 mm pieces and placed in 3 ml of Protocol 2 digestion buffer [2 mg/ml collagenase type IV (Worthington Biochemical Corporation) and 1.2 U/ml dispase II (Sigma-Aldrich or Thermofisher Scientific) in Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.9 mM CaCl\(_2\)]. Tissue was incubated at 37°C for 15 min with gentle rocking. Following incubation, tissue digestion buffer with tissue clusters was triturated by pipetting 12 times using a 10 ml serological pipette. Dishes were again incubated at 37°C and triturated twice more (45 min of total digestion time). The final trituration was conducted by pipetting 30 times with a p1000 pipette. For Protocol 3, isolated mouse hearts were finely minced using forceps, placed in 10 ml Protocol 3 digestion buffer [125 U/ml collagenase type XI (Sigma-Aldrich), 60 U/ml hyaluronidase type I-s (Sigma-Aldrich), and 60 U/ml DNase 1 (Sigma-Aldrich) in DPBS supplemented with 0.9 mM CaCl\(_2\) and 20 mM HEPES] incubated at 37°C for 1 hour with gentle agitation, triturated 20 times using a 10 ml serological pipette, and placed on ice. All cell suspensions were filtered using a 40 \(\mu\)m cell strainer. Filtered suspensions were placed into 50 ml tubes with 40 ml of DPBS and centrifuged at 200 g for 20 min with centrifuge brakes deactivated to remove small tissue debris. Cell pellets were resuspended in 250 \(\mu\)l 2% FBS/HBSS solution before staining with various antibodies and reagents for flow cytometry or FACS.

**Antibody, nuclear and metabolically active cell staining for flow cytometry.**

Antibody staining for specific antigens (Online Table I) was conducted in 100 \(\mu\)l of single cell suspension (in 2% FBS/HBSS) using Protocols 1, 2, or 3 after FC receptor blocking with CD16/CD32 antibody. Following 1 hour antibody incubation at 4°C, calcein (calcein-AM or calcein-violet; Life Technologies) and/or Vybrant® DyeCycle™ Orange (VDO; Life Technologies) were added to antibody/cell suspensions at final concentrations of 5 and 2.5 \(\mu\)M, respectively. Samples with dyes added were incubated in a 37°C water bath for 10 min, before placing samples on ice. Samples were washed with 2% FBS/HBSS and
resuspended in 2% FBS/HBSS with or without the viability dyes DAPI or 7-AAD. All flow cytometry was conducted on LSR II Fortessa Flow Cytometers (BD Biosciences) or LSR II Flow Cytometers (BD Biosciences). For compensation of fluorescence spectral overlap, UltraComp eBeads (eBioscience, Inc.) were used following the manufacturer’s protocols. FCS 3.0 files generated by flow cytometry were initially processed using FlowJo Software (Tree Star, Ashland, USA) for automated compensation. Dye-positive or negative cell populations were gated and exported as new FCS 3.0 files and uploaded to Cytobank Premium for subsequent SPADE analyses. For analysis of total cardiac cells (Figure 2), metabolically active (calcine \(^\text{\textsuperscript{\textdagger}}\)), nucleated (VDO \(^\text{\textdagger}\)), and viable (DAPI \(^-\)) events were gated as shown (Figure 2A). Events were gated on viable (7-AAD \(^-\)) single cells, before gating on CD31\(^\text{\textdagger}\)CD45 \(^-\) or CD45 \(^+\) events for analysis of endothelial cells and leukocytes, respectively (Figure 3A-F). Events were gated on calcine \(^+\), viable (7-AAD \(^-\)), CD31\(^\text{\textdagger}\)CD45 \(^-\) events for analysis of resident mesenchymal cells, RMC, (Figure 3G-J; Online Figures V and VI). All SPADE analyses were conducted with the target number of nodes set at 200 or 100 and down-sampled events target set at 100%.

**Statistical analyses.**

All statistical analyses were conducted using Prism 6 for Windows software (GraphPad Software, Inc., La Jolla, USA). Statistical variability expressed as means ± SD.

See Online Materials and Methods for additional information.

**RESULTS**

**Immunohistochemical analysis of cardiac cellular composition.**

To determine the proportions of the major types of non-myocyte cells in the adult murine heart, we conducted histological analysis of cardiac tissue from adult Cx3cr1GFP/\(^+\) mice\(^10\), which express GFP in monocytes, macrophages, and dendritic cells including subsets of cardiac tissue macrophages\(^11\), 12. In addition to GFP, we stained tissue sections with a hematopoietic lineage antibody cocktail (Lin 1) targeting leukocyte antigens: CD45, Mrc1, B220 and MHC class II, to distinguish a broad cross-section of cardiac leukocytes (Online Figure IA). Cardiomyocytes and endothelial cells were identified by wheat germ agglutinin (WGA) and isolectin B4 (IB4), respectively. Using this combination of reagents, we were able to examine regional differences in cardiac cellular composition (cardiomyocytes, endothelial cells, and leukocytes) in both ventricles and the interventricular septum by immunohistochemistry (Figure 1A-B, Online Figures IA and IIA-C). We found approximately 31.0 ± 4.2% of nuclei were cardiomyocytes, 43.6 ± 4.1% endothelial cells, and 4.7 ± 1.5% leukocytes. These proportions remained similar in all regions of the heart (Online Figure IIB). Unmarked cells accounted for approximately 20.7 ± 4.5% of nuclei. When considering only non-myocytes (Online Figure IIC), our analysis indicates that approximately 63.3 ± 5.4% are endothelial cells, 6.8 ± 2.1% are leukocytes, and 29.9 ± 5.9% were unmarked.

As a number of studies have suggested that fibroblasts constitute the majority of non-myocytes in the heart, similar analyses were performed in cardiac tissues from mice containing either a nuclear localized H2B-eGFP expressed from the PDGFR\(\alpha\) locus\(^13\), PDGFR\(\alpha\)\(^\text{\textsuperscript{\textdaggerpp}}\), (Figure 1C and Online Figure IB) or GFP driven by a Collal promoter (3.2 kb) and HS 4,5 enhancer (1.7 kb), Collal-GFP\(^14\). These two mouse lines drive expression of GFP in both epicardial and endocardial lineages of fibroblasts\(^15\)-17. Using these two strains and focusing on the left ventricle, we independently stained for cardiomyocytes (ACTN2), endothelial cells (IB4 or DACH1), hematopoietic lineage cocktail (Lin 2) which contains anti-CD5, -CD11b, -B220, -7-A, -Gr-1 (Ly6G/C), and -Ter-119 antibodies, and vascular smooth muscle cells (VSMCs)/pericytes\(^18\) (anti-PDGFR\(\text{\textsuperscript{\textdagger}}\)) (Figure 1C, Online Figure IB, and data not shown). Approximately 32.6 ± 4.8% of nuclei corresponded to cardiomyocytes, 55.0 ± 6.0% to IB4\(^+\) endothelial cells, 5.7 ± 1.1%
to VSMCs/pericytes, 8.5 ± 1.5% to leukocytes, and 12.6 ± 2.5%, and 12.7 ± 3.6% to fibroblasts (PDGFRαGFP/+ and Coll1a1-GFP mice, respectively; Figure 1D). Because the endothelial values were considerably higher than previous reports, expression of DACH1 was used as an independent marker for endothelial cells (Figure 1C and D). DACH1 is expressed in the nuclei of coronary vessel endothelial cells (Online Figure 1B) but not in endocardial cells and therefore, was a reliable marker for quantification of coronary endothelial cells. Figure 1C illustrates the frequency of endothelial cells and fibroblasts relative to other nuclei. DACH1+ endothelial cells constituted 51.8 ± 3.9% of the nuclei. The sum of these data may be greater than 100%, due to inaccuracies in attributing nuclei to cells stained for plasma membrane components. Nonetheless, these independent data sets demonstrate that endothelial cells are the major cell type in the adult murine ventricle. Surprisingly, these results also show that fibroblasts, marked by two unrelated mouse GFP reporter lines, contributed to <20% of total non-myocyte nuclei.

To determine whether human tissue mirrors these findings, we analyzed healthy, adult human heart samples, using antibodies that recognize cardiomyocytes, endothelial cells, and leukocytes (Figure 1E and Online Figures IC and IID). Unfortunately, none of the antibodies surveyed consistently delineated human resident mesenchymal cells (RMC). Examination of human tissues demonstrated that 31.2 ± 5.6% of nuclei correspond to cardiomyocytes (ACTN2), 53.8 ± 6.4% to endothelial cells (CD31) and 2.8 ± 1.2% to leukocytes (CD45). DACH1 also identified human endothelial cells, and 51.1% ± 2.9% of the nuclei in the human heart were DACH1+ (Online Figure IID). Thus, estimations of endothelial abundance in the human heart reflect those observed in the murine heart.

Flow cytometry analysis of nucleated metabolically active cells.

We next performed flow cytometry analysis of single cell preparations as an alternative approach for evaluating cardiac cellular composition. When using conventional methods, a number of technical issues limit the accurate assessment of relative cell proportions by flow cytometry. These issues include the ability to clearly discriminate viable, nucleated cells from tissue debris and cell clusters. Normally, this issue is mitigated by using light-scattering properties of cells such as forward- and side-scatter (FSC and SSC). However, such approaches can skew data regarding the relative proportions of cell types. Therefore, for analysis of relative proportions of broad cardiac non-myocyte cell types (endothelial cells, leukocytes, and RMC), we developed a reliable approach for resolving nucleated cells from tissue debris using Vybrant® DyeCycle™ Orange (VDO), calcein AM (metabolically active cells) and DAPI (cells with compromised membranes) (Online Figure IIIA). To determine the correct gating position for nucleated VDO+ cells within the DAPI−, calcein+ gated population (Online Figure IIIB), we isolated and analyzed cells from 4 regions (R1-4) with differential VDO labeling (Online Figure IIIB-D) by fluorescence activated cell sorting (FACS). Microscopy showed that R1 and R4 contained predominantly small cell fragments or clusters, whereas R2 and R3 contained mainly nucleated single cells. Analysis of FSC-H and FSC-A parameters confirmed R1 and R4 comprise small and large elements, respectively; R2 and R3 were cells with a narrower size distribution (Online Figure IIC). Further analysis of R1 events showed that almost all of these entities are CD31+ and CD105+ (data not shown), indicating that a potentially significant proportion of these entities may be non-nucleated endothelial microparticles (EMPs) or apoptotic bodies. These results point to reliable criteria for distinguishing dead cells and debris from viable, single cells. However, it should be noted the VDO component could be replaced by conventional singlet gating with some contamination of R1 and R4 elements. In consideration of the enhanced accuracy conferred by the incorporation of the nuclear-staining dye, we have employed it for flow cytometric survey of all nucleated non-myocytes in the myocardium.

We used this approach to identify a tissue dissociation protocol that yields the greatest number of viable, nucleated cells. Three cardiac single cell suspension protocols with distinct dissociation enzyme cocktails were compared: Protocol 1, Protocol 2, and Protocol 3. The total number of nucleated cells isolated per milligram of tissue was 4241.8 ± 517.4, 11223.3 ± 1194.3, and 6728.9 ± 491.7 for Protocols 1,
2, and 3 respectively (Online Figure IVA). All three protocols demonstrated >95% calcein labeling in nucleated/viable cells (Online Figure IVB). Given the high yield of nucleated/viable cells, we used Protocol 2 for subsequent analyses. It should be noted that during the process of protocol screening we assessed Langendorff perfusion for cell isolation using several commonly referenced digestion cocktails. We found that these methods yielded fewer viable non-cardiomyocytes and contained contamination by valvular interstitial cells (data not shown).

Two identification methods were used to determine if mural cells (such as VSMCs and pericytes) could be recovered from Protocol 2 isolates. To mark VSMCs, we employed a lineage tagging system in a transgenic mouse that expresses Cre controlled by an $SMA$ promoter ($SMACreERT2$) and a Cre reporter ($ROSA26RtdTomato$). An anti-NG2 antibody was used to identify pericytes. We found that VSMCs and pericytes could be observed after isolation with Protocol 2 (Online Figure V), suggesting that this population of cells will be included when determining relative cardiac cell proportions.

Cardiac non-myocyte cellular composition.

To determine cardiac non-myocyte cell composition, we conducted flow cytometry with antibodies for endothelial cells (CD31 and CD102), leukocytes (CD45 and CD11b) and RMC (CD90 and Sca-1) in addition to nuclear and metabolic activity stains described above. Flow cytometric analyses with manual gating of nucleated, calcein$^+$ cells indicated that CD31$^+$CD45$^-$ cells (endothelial cells) constituted 62.1 ± 3.9%, CD45$^+$ cells (leukocytes) 9.6 ± 1.3%, and CD31$^-$CD45$^-$ cells (RMC) 27.3 ± 5.3% of all non-myocytes (Figure 2A).

To better define cell populations represented by this antibody panel (Figure 2B), we conducted high-dimensional analysis of our flow cytometry data using the SPADE (Spanning-tree Progression Analysis of Density-normalized Events) algorithm. SPADE utilizes agglomerative clustering to identify groups of cells (referred to as ‘nodes’) that are phenotypically similar based on multiple surface or genetic markers (described in detail previously). Schematically, nodes are represented as colored circles that are interconnected within a dendrogram. Direct connection of one node to another signifies phenotypic similarity between the two nodes, and their size represents the number of cells within the node. Node color may indicate a range of statistical parameters. For example, all nodes within the dendrogram may be colored relative to the expression level of specific markers that define cell populations. This enables systematic annotation and categorization of individual nodes or groups of nodes as specific cell types. After annotation, nodes may be selected to derive statistical data, such as relative cell proportion.

Following generation of SPADE dendrograms for cardiac cells using the aforementioned markers (Figure 2B), dendrogram branches corresponding to leukocytes, endothelial cells, and RMC were manually annotated based on marker expression levels (Figure 2C and D). Branches with high CD45 expression, encompassing nodes and branches with high CD11b (myeloid cells) and CD90 (T cells), were classified as leukocytes (Figure 2D). Similarly, branches expressing CD31, CD102 but not CD45 were identified as endothelial cells. Remaining nodes, including nodes with high expression of commonly used fibroblast/mesenchymal markers, Sca-1 and CD90, were classified as RMC. Statistical analysis of SPADE branches identified 63.9 ± 3.4% of cardiac cells as endothelial cells, 9.4 ± 1.6% as leukocytes and 26.7 ± 4.0% as RMC (Figure 2E). These values were consistent with histological analyses described above. Moreover, the SPADE data is consistent with the flow cytometry analyses conducted on nucleated cardiac cells manually gated for endothelial cells (CD31$^+$CD45$^-$ cells), leukocytes (CD45$^+$), and RMC (CD31$^-$CD45$^+$) (Figure 2A). Together these findings confirm that endothelial cells are the most prevalent cell type in the adult murine heart.
**Endothelial and leukocyte diversity.**

To determine the diversity of cardiac endothelial cells and leukocytes, we also conducted SPADE analysis following gating on viable, nucleated CD31+ CD45+ endothelial cells or CD45+ leukocytes. Endothelial cells were clustered based on surface expression of CD102, CD105, podoplanin, and CD90. We found 94.3 ± 0.7% corresponded to vascular endothelial cells (VEC; CD102+ CD105+ nodes) and 5.2 ± 0.7% corresponded to lymphatic endothelial cells (LEC; podoplanin- nodes) (Figure 3A-C). To characterize leukocyte subsets, we clustered CD45+ cells based on surface expression of CD11b, CD64, B220, IgM, CD3ε, and CD90 (Figure 3D and E). Myeloid cells (CD11b+ nodes), B cells (CD11b B220+ nodes), T cells (CD11b CD3ε+ nodes), and non-myeloid/lymphoid (CD11b B220 CD3ε- nodes) cells were clearly identified within dendrograms. Consistent with cell identity, CD64, IgM, and CD90 were highly expressed in nodes corresponding to myeloid cell, B cell, and T cell clusters, respectively. Statistical analysis showed 81.4 ± 1.4%, 8.9 ± 0.6%, 3.1 ± 0.4% and 6.6 ± 0.6% of leukocytes corresponded to myeloid cells (CD11b+), B cells (B220+), T cells (CD3ε+), and non-myeloid/lymphoid cells (CD11b B220 CD3ε-), respectively (Figure 3F).

**RMC composition.**

While endothelial cells and leukocytes can be identified using a variety of antibodies that recognize surface proteins, reagents for classifying fibroblasts and mural cells (VSMC and pericytes) are limited. Consequently, we attempted to identify uniform and specific antibodies suitable for flow cytometric identification of these two populations. In the murine context, an anti-PDGFRβ antibody would be a candidate for mural cells. Flow cytometry demonstrated that the PDGFRβ monoclonal antibody, APB5, did not label primary cells to a satisfactory level (data not shown for six independent sources), although the same monoclonal antibody can be used for immunohistochemistry.

Unlike the mural cell population, we were able to clearly identify the proportion of RMC that were cardiac fibroblasts by using genetically-tagged Coll1α1-GFP, PDGFRαGFP/− and Coll1α1-GFP; PDGFRαGFP/− transgenic mice that express GFP in resident fibroblasts as described above. By gating upon the CD45 CD31 population, we observed that more than half of the RMC were GFP+ (Figure 3G and H). To determine whether GFP cells from Coll1α1-GFP and PDGFRαGFP/− mice represent similar cardiac cell populations, we conducted SPADE analysis by clustering on CD31, CD102, CD45, CD11b, CD90 and Sca-1. A high degree of overlap occurred when comparing dendrograms for Coll1α1-GFP and PDGFRαGFP labeled cells (Online Figure VI).

While cells from the two GFP fibroblast mouse lines were convenient for categorizing and surveying the fibroblast population, use of these transgenic mice as a universal tool to analyze fibroblasts is not always feasible. Because antibodies validated for flow cytometry were readily available for three commonly used fibroblast surface antigens, Sca-1, CD90, and PDGFRα, we surveyed these by flow cytometry. PDGFRα antibodies were assessed on NIH3T3 fibroblasts, mouse embryonic fibroblasts (MEFs), including PDGFRα null MEFs29, and primary cardiac single cell isolates from wild type and PDGFRαGFP/− mice. None of the PDGFRα antibodies examined clearly and uniquely distinguished a positive cell population (Online Table I and data not shown), although PDGFRα expression was observed in cardiac fibroblasts by immunohistochemistry (Online Figure VII). Interestingly, following unsupervised SPADE clustering of RMC from Coll1α1-GFP mice, we found that most GFP+ nodes were also MEFSK4+ (Figure 3I). Notably, similar correlation of gene expression within these nodes was not observed for CD90 or Sca-1. The MEFSK4 antibody has been used to identify and remove MEFs during culture of induced pluripotent stem cells and embryonic stem cells. This antibody identifies MEFs from C57BL/6, CF1, CF6, and DR4 mouse strains and an epitope on NIH3T3 cells30. The close correlation of GFP expression and MEFSK4 detection was further underscored when comparing GFP expression to MEFSK4, CD90, or Sca-
profiles (Figure 3J). Indeed, almost all GFP\(^+\) RMC from Col1a1-GFP mice, PDGFR\(\alpha\)\(^{GFP/+}\) mice, or Col1a1-GFP;PDGFR\(\alpha\)\(^{GFP/+}\) double transgenic mice were MEFSK4\(^+\), and most MEFSK4\(^+\) RMC were GFP\(^+\) (Online Figure VIII). MEFSK4 antigen expression was independent of activation status, as surface staining was not decreased after TGF\(\beta\)1 stimulation of MEFs or after myocardial infarction (data not shown). Therefore, this population must be excluded from analysis before considering the fibroblast lineage by flow cytometry. We have not been successful in tissue staining with the MEFSK4 antibody. To further investigate the MEFSK4\(^+\) RMC population by flow cytometry, we co-stained for the pericyte marker, NG2. NG2\(^+\) cells express the MEFSK4 epitope at lower levels than GFP\(^+\) cells, and NG2\(^+\) cells account for a majority of the MEFSK4\(^+\) GFP RMC population (Online Figure IX). To further define MEFSK4\(^+\) cells, we isolated CD31\(^-\),CD45\(^-\), MEFSK4\(^+\) cells by FACS and found that fibroblast gene expression is enriched in this cell population compared to whole ventricle (Online Figure X). These findings indicate that Sca-1 and CD90 are suboptimal markers of resident cardiac fibroblasts and that MEFSK4 may be a suitable surrogate marker for cardiac fibroblasts.

**Cardiac fibroblast diversity.**

To further examine the spectrum of fibroblasts labeled by PDGFR\(\alpha\)\(^{GFP}\), Col1a1-GFP and MEFSK4, we performed lineage tracing by intercrossing Tcf21\(^{Cre/+}\); ROSA26\(^{tdTomato}\) mice with the fibroblast GFP-expressing mouse lines. In the Tcf21\(^{Cre}\) mouse model, resident fibroblasts and their descendants are indelibly tagged with tdTomato fluorescence following Cre induction\(^ {15,31}\). We induced Cre-mediated recombination at either embryonic day 16.5 (Tcf21\(^{Cre/+}\);ROS26\(^{tdTomato}\);Col1a1-GFP) or in the adult (Tcf21\(^{Cre/+}\); ROSA26\(^{tdTomato}\);PDGFR\(\alpha\)\(^{GFP/+}\)) and conducted flow cytometry 2-6 months after induction to determine the proportion of tdTomato\(^+\) cells (cardiac fibroblasts) that were GFP\(^+\). Gating on the tdTomato\(^+\) cells revealed that ~95% were positive for GFP and MEFSK4 (Figure 4 and data not shown), demonstrating that cardiac GFP\(^+\) cells from PDGFR\(\alpha\)\(^{GFP/+}\) and Col1a1-GFP mice comprise Tcf21 lineage fibroblasts. Taken together, these data suggest that the population of cells identified as fibroblasts is relatively uniform as determined by four independent markers (PDGFR\(\alpha\)\(^{GFP/+}\), Col1a1-GFP, MEFSK4 and Tcf21 lineage). Furthermore, our analysis indicates that this cell population constitutes 15% of the non-myocyte cell pool, equating to ~11% of the total cells of the heart when assuming ~30% of the cells are cardiomyocytes.

**DISCUSSION**

A comprehensive understanding of cardiac cellular composition will guide development of therapeutics that promote heart repair and regeneration. To date, attempts to survey the cellular composition of the heart, even in model organisms such as the mouse and rat, have been hindered by difficulties in cell identification and isolation. Here, using recently developed techniques and genetic tools, we demonstrate that endothelial cells outnumber other cell types in the adult mouse heart ventricles (Figure 5) and that the cardiac fibroblast population is much smaller than previously reported. Nonetheless, classic fibroblast markers such as CD90 and Sca-1 underrepresent the resident fibroblast population.

While the accuracy and reliability of immunohistochemical data depends on the specificity and sensitivity of the antibodies used, it can be a powerful approach for evaluating cell populations within a tissue. Here, two histological approaches in separate colonies of mice generated highly analogous results that were further corroborated by the analysis of human tissue sections. Using histological analyses, however, we were unable to evaluate the proportion of myocytes relative to non-myocytes with a high level of precision. This is due to the variability and dynamism of the number of nuclei per cardiomyocyte in addition to the differences between rodents and humans—most murine cardiomyocytes are binucleated\(^ {12,33}\).
while most human cardiomyocytes are mononucleated. Therefore, estimations of myocyte to non-myocyte ratios will differ depending on the species and age.

There are a number of explanations as to why our findings regarding the number and proportion of endothelial cells in the heart differ from those of previous studies. First, access to new and enhanced reagents enabled more accurate identification of cell populations than was previously possible. Second, use of an isolation protocol that yielded the greatest number of viable cells at the expense of cardiomyocytes may explain why flow cytometry results strongly correlated with results from immunohistochemical analyses but produced different outcomes compared to previous studies. Many published protocols use digestion conditions that favor the isolation of cardiomyocytes with non-cardiomyocytes being the byproducts of the isolation. Third, establishment of the criteria of cell nucleation and vitality to determine cell frequency enabled us to perform a more accurate analysis. This approach eliminates the dependency on using light-scattering (forward- and side-scatter) properties of cells for identifying viable cells, a practice that could lead to over- or under-representation of cell types and inclusion of tissue debris as cellular events. Fourth, in classifying cell populations we used an unsupervised clustering algorithm (SPADE) following objective cell gating. SPADE and similar clustering algorithms enable superimposition of expression data for multiple cellular markers to visualize and quantify non-myocyte cardiac cell populations. The identity of endothelial cells was confirmed using multiple endothelial markers including CD31, CD102, and CD105, in addition to exclusion of leukocyte markers CD45 and CD11b.

While the recovery of cell populations and flow cytometry data presented here correlates with the cellular distribution documented by immunohistochemistry, the possibility remains that not all cells have been included in the current assessment. These may include cells that are more sensitive to the digestion protocol or are not recovered due to insufficient digestion. Notably, we have not tested the efficacy of the nuclear and metabolic activity (VDO and calcein) staining approach to distinguish between activated, proliferating, and quiescent cell populations.

The discrepancy in leukocyte proportions determined by flow cytometry and immunohistochemistry may be attributed to inefficiencies in antigen detection in fixed tissues. Indeed, this is likely for leukocyte staining using anti-CD45 antibodies, which do not reveal stellate-shaped myeloid cells in fixed human or mouse tissue. Utilizing antibody cocktails for staining mouse cardiac tissue may overcome this issue; nevertheless, immunohistochemical analyses of leukocytes may still fail to detect all cells. By flow cytometry analysis—focusing on nucleated metabolically active cells and SPADE—we have estimated that leukocytes comprise approximately 7-10% of all non-myocytes. The vast majority of these cells were identified as myeloid cells and, in particular, macrophages. A number of lymphocytes were also detected, although at a much lower prevalence.

Histologic and flow cytometric analysis also suggests that the relative number of fibroblasts in the heart may have been over-estimated in the past. The lack of a clear definition for fibroblasts has been at the root of many difficulties in quantifying and tracking these cells in vivo. In some cases, cardiac fibroblasts have been quantified by excluding cells that lack structural elements corresponding to endothelial cells, VSMCs, or cardiomyocytes. According to this definition, pericytes and leukocytes would also be classified as fibroblasts. Another confounding factor is that markers such as DDR2, CD90, Sca1, and vimentin are not unique to fibroblasts, and not all fibroblasts express these proteins. While resident cardiac fibroblasts derive from two embryonic sources, the consensus is that PDGFRα is expressed in both populations. The utility of CD90 for identification of fibroblasts, on the other hand, remains a point of debate. Previously, we have shown that cardiac Tcf21 lineage cells, and GFP+ cells from PDGFRαGFP and Col1a1-GFP mouse hearts have similar levels of fibroblast gene expression including Col3a1, Col6a1, Den, and MMP2 with lack of expression of smooth muscle, endothelial, and cardiomyocyte genes. As flow cytometric results demonstrate that the MEFSK4 antibody detects Tcf21 lineage cells, and GFP+ cells from PDGFRαGFP and Col1a1-GFP mouse hearts, and surface staining by the MEFSK4 antibody correlates with GFP-expressing
cells when excluding leukocytes, this antibody can be used to identify cardiac fibroblasts by flow cytometry when use of the genetic systems is not feasible. Our results also suggest that CD90 and Sca1 only capture a fraction of the resident fibroblasts. This analysis represents the most comprehensive estimation of cardiac fibroblasts to date.

Recent reports have proposed that pericytes contribute significantly to the cardiovascular remodeling process. In the uninjured heart, we show that these cells account for approximately 5% of non-cardiomyocytes and that they also express MEFSK4, albeit at lower levels compared to fibroblasts. Further investigation will be required to determine MEFSK4 expression in the activated pericyte population. The establishment of these cellular proportions at baseline will pave the way for future work examining how these populations vary in the postnatal, aged, and injured heart.

Although the role of the endothelial cell as a dynamic regulator of tissue responses is increasingly recognized, their abundance and roles in the heart are commonly underappreciated. The proximity of endothelial cells to the coronary circulation and cardiomyocytes provides an access point for therapeutic manipulation. Therefore, a more comprehensive understanding of these potential cellular interactions will be required. Taken together, our findings redefine the cellular composition of the adult murine and human heart and point to the cardiac endothelial cell as a potentially important protagonist in cardiac homeostasis, disease, and aging.

**SOURCES OF FUNDING**

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**DISCLOSURES**

None.
REFERENCES

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FIGURE LEGENDS

**Figure 1.** Quantification of major cardiac cell types. (A) Representative confocal microscopy optical section of cardiac tissue stained from a 10 week old Cx3cr1\(^{GFP/+}\) mouse. (A’) Magnified view of image shown in A. Nuclei (DAPI); cardiomyocyte boundaries (wheat germ agglutinin, WGA); endothelial cells (isolectin B4, IB4); and leukocytes (Lin1 cocktail: GFP, CD45, Mrc1, MHCII and B220). Arrowheads indicate a representative cardiomyocyte (CM), endothelial cell (EC), leukocyte (Lin1\(^+\)) and unstained cells (unst.). 0.969 µM optical sections. (B) Average proportions from all myocardial regions examined (48 micrographs from 4 hearts). (C) Representative fluorescence microscopy image of cardiac tissue from a PDGFR\(^{α}GFP/\) mouse stained for DACH1. Arrowheads indicate a representative endothelial cell (EC) and fibroblast (FB). (D) Alternative quantification of cells in mouse left ventricle (LV) with identification of VSMCs/pericytes and fibroblasts. Lin2 cocktail (CD5, CD11b, B220, 7-4, Gr-1, and Ter-119): leukocytes; PDGFRB: VSMCs/pericytes; IB4: endothelial cells; DACH1: endothelial cell nuclei; actinin 2 (ACTN2): cardiomyocytes; Coll1α-GFP or PDGFRα\(^{GFP/+}\): fibroblasts. (E) Quantification of ACTN2\(^+\) cardiomyocytes; CD31\(^+\) endothelial cells and CD45\(^+\) leukocytes in human heart tissue. Data averaged from all quantified myocardial regions (ventricular septum, and left and right ventricles). Tissue from 3 independent, adult, healthy, human heart samples.

**Figure 2.** Flow cytometry analysis of cardiac composition. (A) Gating of nucleated (VDO\(^+\)), viable (DAPlow), and metabolically active (calcein\(^+\)) cells with subsequent distribution based on CD31 and CD45 expression. (B) Expression of cell surface markers. (C) Unsupervised cell clustering using SPADE analysis following gating on VDO DAPI\(^low\)calcein\(^+\) events. Cell clustering based on CD31, CD102, Sca-1, CD45, CD90 and CD11b expression. Dendrogram node color represents relative expression as indicated by heat map. (D) SPADE dendrogram with endothelial cells (ECs), leukocytes (Leuks), and resident mesenchymal cells (RMC) manually annotated. Clustering based on CD31, C102, Sca-1, CD45, CD90 and CD11b expression, following manual gating on nucleated, calcein\(^+\) DAPI\(^low\) cells. (E) Proportions of cells within branches corresponding to ECs, Leuks or RMC identified in the SPADE dendrogram in D (n=8). Heat map indicates level of expression.

**Figure 3.** Major cardiac cell subsets. (A) Representative gating of cardiac CD31\(^-\)CD45\(^-\) cells for SPADE analysis. (B) Unsupervised SPADE clustering of CD31\(^-\)CD45\(^-\) cells. Cell clustering based on CD102, podoplanin, CD105 and CD90 expression. (C) Proportions of VECs and LECs of total CD31\(^-\)CD45\(^-\) cells. (D) Gating of cardiac CD45\(^+\) cells for SPADE analysis. (E) Unsupervised SPADE clustering of CD45\(^+\) cells. Cell clustering based on CD11b, CD64, B220, IgM, CD3e and CD90 expression. MFs, macrophages. (F) Proportions of cardiac leukocytes based on surface marker expression. n=3-4 for 3A-F. (G) Representative gating of cardiac CD31 CD45\(^-\) cells (RMC) for SPADE analysis. (H) Percent of GFP\(^+\) cells in RMC from indicated genotypes (n=6-9). (I) Unsupervised SPADE clustering of RMC from Coll1α-GFP mouse hearts. Cell clustering based on MEFSK4, GFP, CD90, and Sca-1 expression. (J) Contour plots of MEFSK4 staining, CD90, or Sca-1 concomitant with GFP expression in cells from the indicated mice (CD31 CD45\(^-\) population). All above populations were gated following pre-gating on calcine\(^+\) singlet cells that were either DAPI or 7-AAD\(^-\). Heat maps indicate level of expression.

**Figure 4.** MEFSK4 staining and fibroblast GFP expression overlap in Tcf21 lineage fibroblasts. Flow cytometry contour plots displaying gating of Tcf21 lineage cells (tdTomato\(^+\)) from (A) Tcf21\(^{Cre\(+\)}\);ROSA26R\(^{tdTomato\(+\)}\);PDGFRα\(^{GFP/+\}) or (B) Tcf21\(^{Cre\(+\)}\); ROSA26R\(^{tdTomato\(+\)}\);Coll1α-GFP mice with subsequent analysis of MEFSK4 staining and GFP expression. Cell analysis performed a minimum of 2 months following tamoxifen induction. Representative contour plots.

**Figure 5.** Distribution of major non-myocyte cardiac cell types. Combined data demonstrating relative cell numbers determined by flow cytometry with exclusion of cardiomyocytes.

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Novelty and Significance

What Is Known?

- While cardiomyocytes constitute the vast majority of cardiac cell mass, non-cardiomyocytes account for the majority of cells in the heart.

- Non-cardiomyocytes are diverse and include fibroblasts, endothelial cells, leukocytes, smooth muscle cells, and pericytes.

- The prevailing view is that fibroblasts are the most abundant non-cardiomyocyte cell type in the heart.

What New Information Does This Article Contribute?

- Endothelial cells outnumber all other cell types in the heart, comprising greater than 60% of the non-cardiomyocyte cells.

- Fibroblast numbers are lower than previous estimates and constitute less than 20% of the non-cardiomyocyte cells.

Accurate characterization of the cell types found in the heart is essential for understanding cardiac development, homeostasis, aging, and injury responses. This study provides the first comprehensive survey of major cell types found in the human and mouse heart and their relative abundance. We find that endothelial cells are the most abundant cell type in both the mouse and human heart and that fibroblasts comprise a much smaller proportion of non-cardiomyocyte cells than previously thought. In addition, we demonstrate a standardized approach for identifying cell types in the heart that permits simultaneous surveying of multiple non-cardiomyocyte cell populations. The findings of this study fundamentally redefine our understanding of the cellular composition of the heart and may have implications for studies concerned with cardiac cellular biology in a range of developmental and disease contexts.
Figure 1

A, A': DAPI, Lin1, WGA, IB4

B: Murine

C, D: Murine - LV

E: Human

Legend:
- DAPI
- Lin1
- WGA
- IB4
- EC
- CM
- Unst.
- CMs, ECs, Lin1, Unstained
- ACTN2, IBA1, DACH1, Lin2, PDGFRα, GFP, Coll1α1, DACH1
- CD31, CD45
Figure 3

Endothelial cells

A

CD31

CD45

B

CD102

Podoplanin

VEC

LEC

C

% Total CD31^hi CD45^lo cells

CD105

CD90

(VEC)

(LEC)

( podoplanin^hi )

Leukocytes

D

FSC

CD45

E

CD11b

CD64

B220

IgM

CD11b

CD64

Myeloid

Non-myeloid/lymphoid

B cells

T lymphocytes

T cells

F

% Total CD45^lo cells

100

50

0

100

50

0

Myeloid

Non-myeloid/lymphoid

RMC

G

CD31

CD45

H

% RMC

Col1a1

GFP

CD90

Col1a1/PDGFRα

Col1a1

PDGFRα

I

GFP

CD90

MEF2K4

Sca-1

J

MEF2K4

Sca-1

GFP

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Figure 4

A

TdTomato (Tcf21) vs FSC-A

B

TdTomato (Tcf21) vs FSC-A

GFP (PDGFRα)

GFP (Col1a1)
Figure 5

- Endothelial cell 64%
- Vascular endothelial cell 60%
- Lymphatic endothelial cell 3%
- RMC 27%
- Other 12%
- Fibroblast 15%
- Myeloid 7%
- Other Leuk. 0.6%
- T cell 0.3%
- B cell 0.8%
Revisiting Cardiac Cellular Composition

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Online Materials and Methods

**Mice**

Monash University (Melbourne, Australia)- All mice were maintained on the C57BL/6J background in a specific pathogen-free (SPF) facility and fed standard mouse diet *ad libitum*. All procedures conducted were approved by Monash University Animal Research Platform 2 (MARP2) ethics committee. *Cx3cr1*\(^{GFP/+}\) mice\(^1\) were backcrossed over 10 generations to C57BL/6J and analyses were performed using 10 week old males. University of Hawaii (Honolulu, HI, USA)- Mice were maintained in a specific pathogen-free (SPF) facility and fed standard mouse diet *ad libitum* unless tamoxifen chow was used as noted. All procedures conducted were approved by University of Hawaii IACUC. All experiments using *PDGFR\(\alpha\)*\(^{GFP}\) and/or *Col1a1-GFP* mice\(^2,3\) were performed using animals 2-7 months of age and of mixed genders. Postnatal day 4 *SMACreERT2Tg;ROSA26R\(^{tdTomato}\) animals\(^4,5\) were induced by gastric injection with 300 \(\mu\)g of tamoxifen (5 mg/mL) (MP Biomedicals, 156738) in sunflower seed oil (Sigma, S5007). *Tcf21\(^{Cre/+}\);ROSA26R\(^{tdTomato}\) mice were induced with tamoxifen either by oral gavage of a timed mating female at embryonic day 16.5 (100 \(\mu\)g/g body weight (20 mg/mL tamoxifen)) or feeding tamoxifen chow for two weeks (Harlan Laboratories, TD.130856) All genetically engineered animals with the exception of *Col1a1-GFP* mice have been backcrossed for a minimum of 7 generations to B6.Cg-Gt(ROSA)26Sor\(^{tm14(CAG-tdTomato)Hze}\)/J (ROSA26R\(^{tdTomato}\)), Jax\([007914]\), and contain the *Nnt* deletion.

**Human material**

Human cardiac tissue was obtained after informed consent and used per Institutional Review Board approval of the University of Hawaii at Manoa (CHS #23245). Human tissues were from mixed gender, healthy individuals age 35-55.

**Antibodies**

See Online Table I for antibody information.

**Immunostaining and microscopy**

The two sets of mouse histological data (those from *Cx3cr1*\(^{GFP/+}\) and the fibroblast GFP lines) were gathered independently of each other.

*Staining and analysis of formaldehyde-fixed 150 \(\mu\)m cardiac sections for confocal microscopy.* Isolation of cardiac tissue, staining, and microscopy were previously described\(^6\). Briefly, mice were euthanized by CO\(_2\) asphyxiation and perfused with PBS and freshly prepared 4% formaldehyde (PFA). After overnight fixation of isolated hearts, 150 \(\mu\)m heart sections were prepared using a vibrating blade microtome (Leica Microsystems). Sections were permeabilized in 0.2% Triton-X-100 (Sigma-Aldrich)/PBS solution, prior to blocking (2% goat serum, 1% BSA, 0.1% cold fish skin gelatin, 0.1% Triton X-100, 0.05% Tween-20, 0.05% sodium azide, 0.01M PBS, pH 7.2). Tissue staining was performed in blocking solution using a range of antibodies and reagents summarized in Online Table 1. Fluorescence microscopy images were obtained using a Leica SP5 confocal laser scanning microscope. For quantification of nuclei corresponding to cardiomyocytes, endothelial cells, leukocytes or non-endothelial cell/leukocytes (unstained) we obtained 0.969 \(\mu\)m optical sections using 40X objective lens. Nuclei were manually counted using Imaris software (Bitplane). The minimum number of nuclei counted per image was 125, with a mean of 269.8 nuclei per field of view. For Figure 1A/B and Online Figure IIB/C, nuclei corresponding to cardiomyocytes, Lin1\(^+\) cells, endothelial cells and unmarked cells were classified according to the following criteria: cardiomyocyte nuclei, nuclei associated with large cell bodies (indicated by large cytoplasmic area outlined by WGA staining); Lin1\(^+\) cell nuclei, nuclei with Lin1 antibody cocktail staining within
nuclei or on surrounding plasma membrane; endothelial cell nuclei, nuclei surrounded by IB4 stained plasma membrane; unmarked nuclei, nuclei that do not conform to any of the criteria above.

**Formaldehyde-fixed 10 µm cryosections – Mouse.** 5-12 week old *Coll1a-GFP* or *PDGFRαGFP/* mice were sacrificed by CO2 asphyxiation, perfused with PBS and fixed using freshly prepared 4% formaldehyde for 1 hour at room temperature. The fixed tissues were submerged in 30% sucrose/PBS for 2 hours followed by embedding with Optimal Cutting Temperature (OCT). 10 µm thick sections were prepared before permeabilization with 0.1% Triton-X-100/PBS for 30 min. Sections were blocked with blocking solution (3% BSA, 1.5% normal donkey serum and 0.1% Triton-X-100 in PBS) and incubated overnight at 4°C with primary antibodies; PDGFRβ (CD140b), isolectin B4-Biotin, sarcomeric-α-actinin (ACTN2), and cell lineage-biotin (Lin2). The sections were then washed and incubated with relevant conjugated secondary antibodies for 1 hour before mounting and imaging with a Zeiss Axiovert 200M microscope. As ACTN2 positive nuclei were considered independent events, cardiomyocyte estimations may be overestimated due to multinucleated cells. When choosing images for quantification those containing large coronary arteries were excluded to avoid over representation of any single cell type. In most sections, a maximum of 2-4 cells were observed that exhibited overlapping expression of GFP and the cell type specific antigen being analyzed. The minimum number of nuclei counted per image was 250 with a mean of 314 nuclei per field of view. n=3 sections from 4-5 hearts.

**Formaldehyde-fixed 10 µm cryosections – Human.** Cardiac tissue was flash frozen and stored at -80°C. Samples were thawed and fixed in 4% paraformaldehyde (PFA) at 4°C for 1 hour, cryoprotected in 30% sucrose/PBS at 4°C for 2 hours, embedded in OCT, and sectioned. Immunostaining was performed on 10 µm sections of human cardiac tissue. Sections were permeabilized in 0.1% Triton X-100 for 15 min, blocked in 5% normal donkey serum for 1 hour and incubated in primary antibody overnight at 4°C. Nuclei were stained by DAPI. Corresponding cardiomyocytes, endothelial cells, and leukocytes were quantified within 10 µm-thick sections imaged with a Zeiss Axiovert 200M microscope. As ACTN2 positive nuclei were considered independent events, cardiomyocyte estimations may be overestimated due to multinucleated cells. The minimum number of nuclei counted per image was 86, with a mean of 149 per field of view. n=4-6 sections from 3 hearts.

**Microscopy of isolated cell populations**

After FACS, cells were plated directly in wells of 96-well tissue culture dishes and imaged 30-60 minutes after plating. Images were acquired using a Nikon eclipse Ti-S widefield fluorescence microscope using a 20X objective lens with appropriate filters. For immunocytochemistry cells were isolated according to protocol 2. Cells from a *SMACreERT2;ROSA26RtdTomato* heart were fixed in 4% PFA for 10 minutes and imaged immediately after DAPI staining and plating. Cells were plated in a 35 mm dish or on a chamber slide coated with attachment factor (Life Technologies, S-006-100) for 30 minutes at 37°C and washed with PBS. 2 hours after plating, cells in the chamber slide were fixed in 4% PFA for 10 minutes. The remaining cells were passaged and fixed at 48 hours. For immunocytochemistry, fixed cells were blocked with 1% BSA in PBS and incubated for one hour at room temperature with primary antibody NG2, kindly provided by Dr. William Stallcup. Cells were washed, stained with secondary antibodies for 30 minutes and imaged with a Zeiss Axiovert 200M microscope.

**Quantitative Real Time PCR (qRT-PCR)**

RNA was isolated from cardiac ventricles of 10-15 week old C57BL6/J mice using TRIzol® LS Reagent (Ambion). Briefly, hearts were perfused with ice-cold 1×HBSS (Gibco) for ~2 min before homogenization of tissue in 1 ml of TRIzol® LS Reagent using a Polytron™ tissue homogenizer (Kinematica). Following
homogenization, RNA was extracted according to manufacturer’s instructions. MEFSK4+ RMC were sorted using a BD Influx Sorter (BD Biosciences) by gating on DAPI-CD45-CD31-MEFSK4+ single cells prepared using Protocol 2 (described above). RNA from MEFSK4+ RMC was isolated using RNAqueous®-4PCR Total RNA Isolation Kit (Thermo Fisher Scientific) according to manufacturer’s instructions, with the addition that MEFSK4+ RMC were directly sorted into lysis buffer. RNA quality and concentration was determined by spectrophotometry using a NanoDrop ND-1000 instrument (Thermo Fisher Scientific). SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) was used to perform reverse transcription as per manufacturer’s instructions. Quantitative PCR analysis was performed on RNA from cardiac ventricle (n=4) or MEFSK4+ RMC (n=3, where each biological replicate refers to a pool of 3 heart ventricles) using LightCycler® 480 SYBR Green I Master Mix (Roche) and a LightCycler® 480 instrument (Roche). The 2−ΔΔCt method was employed for determining relative gene expression levels, using the geometric mean of 3 housekeeping genes for normalization of input RNA: TATA-binding protein (TBP), hypoxantine-guanyl ribosyltransferase (HPRT) and actin-β. Primer sequences used in qRT-PCR gene expression analysis are summarised in Online Table II.

Online References

Online Table I. Cell-specific reagents for tissue/cell staining.

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FC: flow cytometry; TS: tissue staining; ICC: immunocytochemistry

Note that multiple clones of CD90 antibodies were tested with similar results for all clones and company sources.
### Online Table II. Primers used for qRT-PCR.

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</tr>
<tr>
<td>Collagen 1 alpha1 (Col1a2)</td>
<td>Forward: GGCSCCCCTTGGATGACTGCGCT</td>
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<tr>
<td></td>
<td>Reverse: CGCCACGGGGGACCAGCAATC</td>
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<td>Cluster of differentiation 90 (CD90)</td>
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<td></td>
<td>Reverse: CTGGGACACTGCGAAGACTGA</td>
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<tr>
<td>Discoidin domain receptor 2 (DDR2)</td>
<td>Forward: TTCCCTGCCAGCGAGTCGA</td>
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<td></td>
<td>Reverse: ACCATGCAACCCTGACTCCTCA</td>
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<tr>
<td>Tenascin C (Ten C)</td>
<td>Forward: TCCCCGGGACTGTAGCCAGC</td>
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<tr>
<td></td>
<td>Reverse: CGTGCACTGCGGCGAGCG</td>
</tr>
<tr>
<td>Myosin heavy chain 6 (Myh6)</td>
<td>Forward: ACGCCTAGAGGGCGAGACC</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCGGCTCGAAGGCGAGGCG</td>
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<tr>
<td>TATA-binding protein (TBP)</td>
<td>Forward: AGATGTGCCAGGGCGGCTCGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: AAATAGTGTGTGGCGACTGCG</td>
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<tr>
<td>Hypoxantine-guanyl ribosyltransferase (HPRT)</td>
<td>Forward: GCGAGGGAGAGCCTGGGGGCT</td>
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<tr>
<td></td>
<td>Reverse: CATCATGCTAATCAGACGCCTGGG</td>
</tr>
<tr>
<td>Actin-beta (actin-β)</td>
<td>Forward: ACAGAAGCTTCGCTTTGCGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACCCATGCCACCATCACG</td>
</tr>
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ONLINE FIGURE I (A and B)

A

B220

MHCII

CD45

Mrc1

GFP

IB4

DAPI

PDGFRα

DACH1

IB4

B

DAPI PDGFRαGFP IB4

DACH1 IB4
Online Figure I. Representative micrographs of cardiac tissue stained for indicated markers. (A) Representative confocal microscopy optical section (0.969 µm) of cardiac tissue stained with individual leukocyte antibodies used for quantification of leukocytes in Fig 1A-B. (B) Left panel; representative image from 10 µm sections of PDGFRαGFP/+ heart stained with IB4 and DAPI. Right panel, image demonstrating DACH1 nuclear co-staining of IB4+ endothelial vessels in mouse heart. (C) Representative fluorescence micrographs of 10 µm human cardiac left ventricle stained for ACTN2, CD31, and CD45.
Online Figure II. Distribution of major cardiac cell types. (A) Schematic of cardiac sections and fields of view analyzed for enumerating cardiac cell types in Figure 1A-B. Sections isolated from mid point (mid) or apex were imaged at the left ventricle (LV), interventricular septum (IS) or right ventricle (RV) by confocal microscopy before analysis. (B) Bar graphs display proportion of cardiomyocytes (CM), endothelial cells (ECs), leukocytes (Lin1) and unstained nuclei for each locus, or (C) means of total non-myocyte populations derived from images from all loci. n=8 per loci derived from four mouse hearts (2 images/loci/heart). (D) Quantification of cardiomyocytes (ACTN2), endothelial cells (CD31), endothelial nuclei (DACH1) and hematopoietic cells (CD45) in designated heart regions in human samples. n=11-18 images per cell type from 3 human hearts.
Online Figure III. Identification of viable, nucleated and metabolically active cells by flow cytometry. (A) Gating strategy for identification of calcein$^+$ DAPI$^-$ cells and analysis of VDO$^+$ or VDO$^-$ cell populations, and (B) analysis regions 1-4 (R1-4) of VDO-stained elements. (C) Microscopy images displaying sorted cells or clusters from regions 1-4 (R1-4) indicated in B, imaged after plating for 30 min. Data representative of at least two similar experiments. All images captured using 20X objective. (D) Flow cytometry scatter plots of FSC-H and FSC-A of cells with regions 1-4 indicated in B.
Online Figure IV. Determination of optimal tissue dissociation protocol for isolation of viable nucleated cells. (A) Number of viable (DAPI<sup>-lo/-</sup>) nucleated cells (VDO<sup>+</sup>) isolated using Protocols 1-3. (B) Percent of metabolically active (calcein<sup>+</sup>) nucleated cells. Data is representative of multiple experiments. n=4 per protocol.
Online Figure V. Recovery of VSMCs and pericytes. (A-C) Microscopy and immunohistochemistry of cells using dissociation protocol 2. (A) Fluorescence of VSMCs (red) isolated from a tamoxifen-induced SMACreERT2;ROSA26R<sup>tdTomato</sup> heart (aSMAc<sup>Cre</sup>). (B) Isolated adherent cells were stained for pericyte marker (NG2) 2 hours after culture. Fibroblasts (GFP). (C) Pericytes (NG2) after passaging cells obtained from a wild type heart.
Online Figure VI. Clustering of GFP nodes in cardiac cells from Col1a1-GFP and PDGFRαGFP/+ mice. Cardiac cells were isolated from Col1a1-GFP and PDGFRαGFP/+ mice and were clustered using SPADE based on expression of CD31, CD45, CD11b, Sca-1, CD90 and CD102. Cells were pre-gated on calcein⁺7-AAD⁻ single cells.
Online Figure VII. PDGFRα expression in cardiac fibroblasts. Immunohistochemistry for PDGFRα in heart sections from (A) PDGFRα^{GFP/+} and (B) Col1a1-GFP mice.
Online Figure VIII. Correlation of MEFSK4 and GFP expression in cardiac cells of Col1a1-GFP, PDGFRαGFP+/ and Col1a1-GFP;PDGFRαGFP− transgenic mouse RMC. Data derived from flow cytometry data where cell populations were gated on calcein+7-AAD− singlets, which were also CD31+CD45− (n=4).
Online Figure IX. NG2\(^+\) population in RMC. Flow cytometry contour plots displaying NG2 expression in RMC relative to MEFSK4 staining and GFP expression. Representative of two replicates. Cardiac cells isolated from \(PDGFR\alpha^{GFP/+}\) mice.
Online Figure X. Fibroblast gene signature of MEFSK4⁺ RMC. qRT-PCR gene expression analysis of MEFSK4⁺ RMC for fibroblast (PDGFRα, Col1a1, Col1a2, CD90, DDR2, TEN C) and cardiomyocyte (MYH6) genes. Expression levels shown relative to cardiac ventricle (n=4 and 3 biologic replicates for cardiac ventricle and MEFSK4⁺ RMC, respectively). PDGFRα: platelet derived growth factor receptor alpha; Col1a1: collagen 1 alpha 1; Col1a2: collagen 1 alpha 2; CD90: cluster of differentiation 90; DDR2: discoidin domain receptor 2, Ten C: tenascin C; MYH6: myosin heavy chain 6.